Biochem. J. (1989) 264, 753–758 (Printed in Great Britain)

The role of protein kinase C in cholinergic stimulation of insulin secretion from rat islets of Langerhans

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The role of the Ca^{2+}/phospholipid-dependent protein kinase C (PKC) in cholinergic potentiation of insulin release was investigated by measuring islet PKC activity and insulin secretion in response to carbachol (CCh), a cholinergic agonist. CCh caused a dose-dependent increase in insulin secretion from cultured rat islets at stimulatory glucose concentrations ($\geq$ 7 mM), with maximal effects observed at 100 μM. Short-term exposure (5 min) of islets to 500 μM-CCh at 2 mM- or 20 mM-glucose resulted in redistribution of islet PKC activity from a predominantly cytosolic location to a membrane-associated form. Prolonged exposure (> 20 h) of islets to 200 nM-phorbol myristate acetate caused a virtual depletion of PKC activity associated with the islet cytosolic fraction. Under these conditions of PKC down-regulation, the potentiation of glucose-stimulated insulin secretion by CCh (500 μM) was significantly decreased, but not abolished. CCh stimulated the hydrolysis of inositol phospholipids in both normal and PKC-depleted islets, as assessed by the generation of radiolabelled inositol phosphates. These results suggest that the potentiation of glucose-induced insulin secretion by cholinergic agonists is partly mediated by activation of PKC as a consequence of phospholipid hydrolysis.

INTRODUCTION

Cholinergic muscarinic agonists, including the endogenous neurotransmitter acetylcholine (ACh) and its synthetic non-hydrolysable analogue carbachol (CCh), enhance glucose-induced insulin secretion from pancreatic β-cells (Malaisse *et al.*, 1967; Gagerman *et al.*, 1978; Nenquin *et al.*, 1984; Hughes *et al.*, 1987). They do so by interacting directly with specific muscarinic receptors in the β-cell plasma membrane, recently identified as being of a glandular M_2 subtype (Henquin & Nenquin, 1988; Verspohl *et al.*, 1988). However, their mode of action in modulating insulin release is still uncertain. Cholinergic agonists have effects on β-cell membrane potential and ionic conductances (Gagerman *et al.*, 1978; Nenquin *et al.*, 1984; Mathias *et al.*, 1985; Henquin *et al.*, 1988) and they also stimulate hydrolysis of phosphatidylinositol 4,5-bisphosphate in both islets (Best & Malaisse, 1983; Morgan *et al.*, 1985; Hallberg, 1986) and insulin secreting tumour cells (Wollheim & Biden, 1986). Inositol phospholipid hydrolysis results in the generation of inositol 1,4,5-trisphosphate and diacylglycerol (DAG), which can liberate intracellular Ca^{2+} and activate Ca^{2+}/phospholipid-dependent protein kinase C (PKC) respectively, in a variety of cell types (Berridge & Irvine, 1984; Nishizuka, 1984). PKC has been identified in islets and in insulin-secreting cell lines (Tanigawa *et al.*, 1982; Lord & Ashcroft, 1984; Persaud *et al.*, 1989), and its activation *in vitro* by DAG analogues or phorbol myristate acetate (PMA), a tumour-promoting phorbol ester, results in stimulation of insulin secretion (Hubinont *et al.*, 1984; Malaisse *et al.*, 1985; Jones *et al.*, 1985). We have previously reported that prolonged exposure of rat islets of Langerhans to PMA down-regulates total cellular PKC activity (Hii *et al.*, 1987). In the present study we have investigated the effects of PKC down-regulation on the ability of CCh to augment glucose-induced insulin release, in order to evaluate the importance of PKC in the mode of action of cholinergic agonists as insulin secretagogues.

EXPERIMENTAL

Materials

Foetal-calf serum, RPMI 1640 medium and Hanks buffered salts solution were from Gibco (Uxbridge, Middx., U.K.). Collagenase (type XI), bovine serum albumin (fraction V), phenylmethanesulphonyl fluoride, PMA, 4α-phorbol didecanoate (4αPDD), CCh, Nonidet P-40, histone type III-S, diolein, phosphatidylserine, ATP and antibiotics were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Polypropylene chromatography columns and Dowex ion-exchange resin were from Bio-Rad (Watford, Herts, U.K.). [γ-^{32}P]ATP (3000 Ci/mmol), [^{3}H]inositol (129.5 Ci/mmol) and $Na^{125}I$ for insulin iodination were from Amersham International (Amersham, Bucks., U.K.). Leupeptin was obtained from Peptide Institute Inc. (London N.1, U.K.). All other reagents were of analytical grade from BDH (Poole, Dorset, U.K.). Rats (150–200 g) were supplied by Charing Cross Hospital Medical School, London W6, U.K.

Islet isolation and culture

Islets of Langerhans were isolated from fed Wistar rats by collagenase digestion of the pancreas (Bjaaland *et al.*, 1988) and either used directly or cultured overnight in RPMI 1640 (pH 7.4) containing 100 μg of strepto-

Abbreviations used: ACh, acetylcholine; CCh, carbachol; PKC, protein kinase C; PMA, phorbol myristate acetate; 4αPDD, 4α-phorbol didecanoate; DAG, diacylglycerol.

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mycin/ml, 100 units of penicillin/ml and 10% (v/v) foetal-calf serum, in a humidified atmosphere of CO_2/air (1:19). For experiments on PKC down-regulation, islets were cultured in the presence of either 200 nM-PMA or 200 nM of the inactive phorbol ester 4αPDD. After 20–24 h, the islets were harvested and washed three times in a bicarbonate-buffered (pH 7.4) physiological salt solution (Gey & Gey, 1936) supplemented with 2 mM-glucose, 2 mM-$CaCl_2$ and 0.5 mg of albumin/ml.

Insulin secretion

Groups of three islets were incubated at 37 °C in 0.6 ml of the bicarbonate-buffered salt solution containing the test substances of interest. After 60 min, islets were pelleted by brief centrifugation (9000 *g*, 15 s) and the supernatant was removed and stored at −20 °C until determination of insulin content by radioimmunoassay (Jones *et al.*, 1988). For studies of the dynamic regulation of insulin release, groups of 100 cultured islets were loaded on to nylon filters (1 μm pore size) and perifused (37 °C, 1 ml/min) with Gey & Gey (1936) buffer supplemented with 2 mM- or 20 mM-glucose and/or CCh. Fractions were collected at 15 s or 2 min intervals and analysed for insulin content by radioimmunoassay.

PKC purification and assay

After overnight culture in RPMI 1640 alone, or in the presence of either 4αPDD or PMA, groups of 400–500 islets were thoroughly washed, incubated for 5 min with 500 μM-CCh if appropriate, resuspended in 200 μl of buffer A (20 mM-Tris/HCl, 2 mM-EDTA, 0.5 mM-EGTA, 50 μg of leupeptin/ml, 1 mM-phenylmethanesulphonyl fluoride and 0.1% mercaptoethanol) and sonicated (MSE Soniprobe, 4 × 15 s). Membranes were separated from cytosol by centrifugation at 30000 *g* for 30 min at 4 °C, the resulting pellets were dispersed by sonication and enzyme activity was solubilized by incubation for 30 min (4 °C) in the presence of 1% Nonidet P-40. Cytosol and membrane extracts were applied to DEAE-cellulose DE52 columns (0.2 ml), and PKC activity was eluted with buffer A supplemented with 120 mM-NaCl. PKC activity in eluate samples was assayed by measuring incorporation of ^{32}P from [γ-^{32}P]ATP into histone type III-S, as described in detail by Persaud *et al.* (1989).

[^{3}H]Inositol phosphate generation

Islets were cultured overnight in RPMI 1640 in the presence of either 200 nM-PMA or 200 nM-4αPDD, washed thoroughly, and then incubated (1 ml, 37 °C, 2 h) in inositol-free RPMI 1640 supplemented with 125 μCi of *myo*-[2-^{3}H]inositol/ml and the appropriate phorbol ester (200 nM). Islets were washed three times with bicarbonate-buffered physiological salt solution (4 °C) containing 10 mM-LiCl, 2 mM-glucose and 1 mM-inositol (unlabelled). Groups of 250 islets were incubated for 5 min at 37 °C in 600 μl of the physiological buffer containing 2 mM- or 20 mM-glucose, 1 mM-inositol and 10 mM-LiCl, in the absence or presence of 500 μM-CCh. Incubations were terminated by addition of 3 ml of ice-cold chloroform/methanol (2:1, v/v), followed by thorough vortex-mixing (1 min) and sonication (4 × 15 s). Aqueous and organic phases were separated by centrifugation (1000 *g*, 5 min) and the upper aqueous phase was removed, mixed with 2 ml of water and applied to a polypropylene column containing Dowex AG1-X8 (200–400 mesh; formate form) for ion-exchange chromatography (Best & Malaisse, 1983). Free inositol and inositol phosphates were eluted from the columns by the stepwise addition of ammonium formate, as previously described (Berridge *et al.*, 1983). Free inositol was eluted with water, after which inositol monophosphates (IP_1), inositol bisphosphates, and more phosphorylated forms, including inositol trisphosphates (IP_3), were eluted with ammonium formate at concentrations of 0.2 M, 0.4 M and 1.0 M respectively. Radioactivity in column eluates and in the organic phase was determined by liquid-scintillation counting.

Statistical analysis

All results are expressed as means ± S.E.M. Differences between means were analysed by Student's paired or unpaired *t* tests or by analysis of variance, as appropriate, and considered significant when $P < 0.05$.

RESULTS

Effects of CCh on insulin secretion

Freshly isolated islets showed considerable variability in their responsiveness to CCh, as has been previously reported for the endogenous muscarinic agonist, ACh (Hughes *et al.*, 1987). Because of this, all further experiments with this agonist used islets which had been maintained in culture medium for 20–24 h, which restored the sensitivity of islets to CCh. As shown in Fig. 1(*a*), the potentiating effect of CCh on glucose-induced insulin secretion was concentration-dependent, with maximal effects being obtained with about 100 μM of the agonist. CCh enhancement of insulin secretion was also dependent on glucose concentration, with significant stimulatory effects being observed only when glucose was present at a concentration of 7 mM or higher, as shown in Fig. 1(*b*). Perifusion of islets revealed that potentiation of secretion by CCh was a result of increase in both first and second phases of glucose-induced (20 mM) insulin release (Fig. 2). Note also, consistent with the results obtained in static incubations, that at a sub-stimulatory concentration of glucose (2 mM), CCh had no significant effect on insulin secretion from perifused islets.

Effects of CCh on PKC translocation

Subcellular fractionation of islets revealed that in the unstimulated state (2 mM-glucose) most of the DE-52-purified PKC activity was associated with the cytosolic fraction (Table 1), and that the PKC activity was redistributed within the cells in response to CCh. Thus at 2 mM-glucose 4.4 ± 2.0% ($n = 5$) of total PKC activity was associated with the membrane fractions. Exposure of islets to 20 mM-glucose for 5 min did not result in redistribution of PKC activity (4.1 ± 1.5% of total activity associated with membranes). However, incubation of islets with 500 μM-CCh for 5 min, a concentration and time course over which CCh enhanced glucose-stimulated insulin secretion (see Figs. 1 and 2), resulted in significant increases in membrane-associated PKC activity in the presence of either 2 mM- or 20 mM-glucose (2 mM-glucose + 500 μM-CCh, 23 ± 2.5% of total; 20 mM-glucose + 500 μM-CCh, 16 ± 2.1% of total; $P < 0.05$ versus 2 mM-glucose and 20 mM-glucose respectively). Note, however, that secretory responses to CCh were only observed in the presence of stimulatory concentrations of glucose (Figs. 1 and 2).

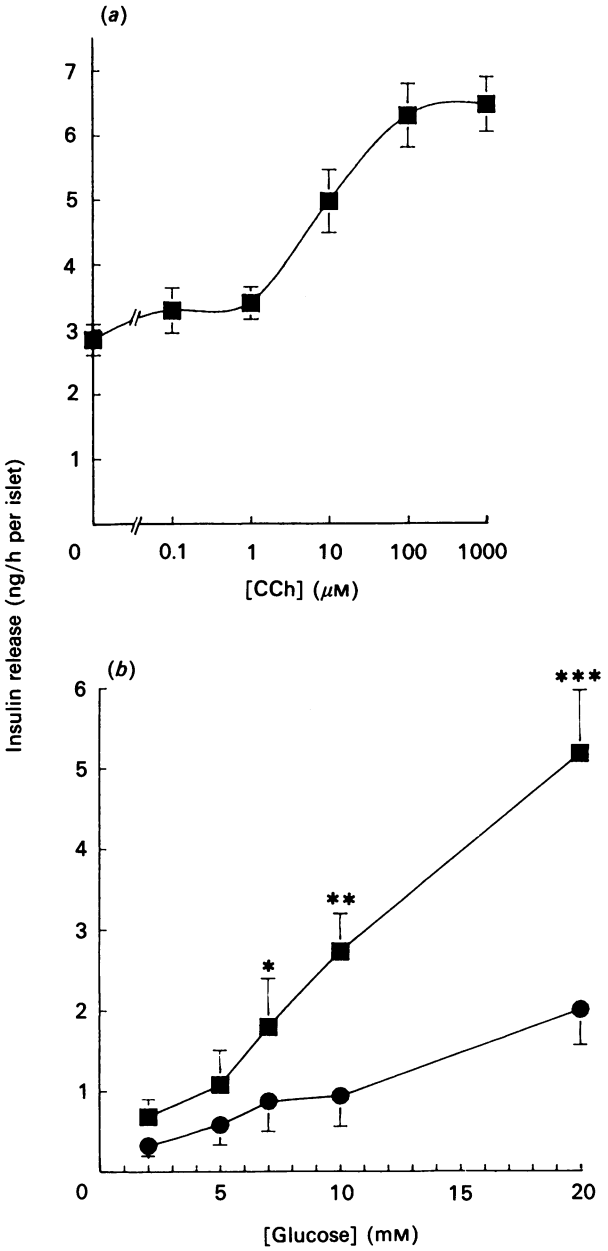

Fig. 1. Potentiation of glucose-stimulated insulin release by CCh

(*a*) Increasing concentrations of CCh caused a dose-related augmentation of 20 mM-glucose-induced insulin secretion. Maximal potentiation was obtained with 100 μM-CCh. Values are means ± S.E.M. of 7–8 observations. (*b*) Curves show insulin release (means ± S.E.M. of 3–4 separate experiments, each of 7–13 observations) in response to increasing concentrations of glucose, in either the absence (●) or the presence (■) of 500 μM-CCh. In all experiments with cultured islets, CCh potentiated insulin release when glucose was present at a concentration of 7 mM or greater. CCh significantly stimulated secretion at 5 mM-glucose in only one experiment (out of three), and in four experiments it had no effect on secretion at 2 mM-glucose. Significance of difference: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus appropriate glucose concentration.

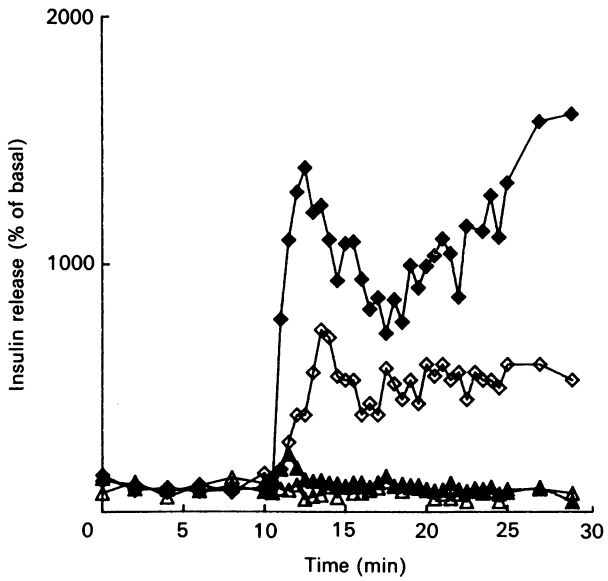

Fig. 2. Time course of CCh effects on secretion

Islets were perifused for 10 min with a medium containing 2 mM-glucose, after which the perifusates contained either 2 mM- (△, ▲) or 20 mM- (◇, ◆) glucose in the absence (△, ◇) or the presence (▲, ◆) of 500 μM-CCh. CCh potentiated secretion at 20 mM-glucose in a biphasic manner and caused a very small, short-lived (< 2 min duration), increase in secretion at 2 mM-glucose. Each treatment is the mean of two experiments.

Down-regulation of PKC activity

Prolonged treatment of islets with PMA (200 nM) virtually abolished cytosolic PKC activity, whether assayed in the presence of diolein or PMA, but caused no detectable change in the low levels of membrane-associated activity (Table 1). Similar treatment with the inactive phorbol ester 4αPDD had no effect on cytosolic or membrane PKC activity. The stimulatory effects of CCh

Table 1. Down-regulation of islet PKC activity by prolonged exposure to PMA

Treatment of islets with 200 nM-PMA for 21 h virtually abolished cytosolic PKC activity, whether measured in the presence of Ca^{2+}/phosphatidylserine (PS)/diolein or Ca^{2+}/PS/PMA, without causing detectable change in the low levels of membrane-associated activity. Each column-eluate sample was assayed in triplicate in the absence and presence of PS/diolein (or PS/PMA), and the difference in ^{32}P incorporation between these conditions was taken to reflect PKC activity in the sample. Levels of significance by unpaired *t* tests: $*P < 0.005$ versus appropriate 4αPDD-treated cytosolic PKC activity ($n = 3$ for all experiments).

	PKC activity (fmol/min per islet)	
	4αPDD-treated	PMA-treated
Ca^{2+}/PS/diolein		
Cytosol	316 ± 20	46 ± 8*
Membrane	33 ± 10	38 ± 5
Ca^{2+}/PS/PMA		
Cytosol	472 ± 59	25 ± 4*
Membrane	44 ± 6	40 ± 9

Table 2. Insulin release from normal and PKC-depleted islets in response to CCh and PMA

Insulin release from five separate experiments (7–9 observations each) is presented as a percentage of the secretion obtained in the presence of 20 mM-glucose alone, because of inter-experimental variation in the absolute amounts of insulin secreted in response to stimuli. Islets exposed to PMA for > 20 h did not respond to a subsequent exposure to PMA, and showed decreased secretory responses to both 500 μM- and 5 mM-CCh. Secretion in response to 20 mM-glucose was not affected by PMA treatment (4αPDD-treated, 1.7±0.4 ng/h per islet; PMA-treated, 2.3±0.4; $n = 5$, $P > 0.2$). Levels of significance by paired t tests: ns, not significant, * $P < 0.05$, ** $P < 0.01$, versus appropriate 20 mM-glucose.

	Insulin release (% of 20 mM-glucose-induced)	
	4αPDD-treated	PMA-treated
2 mM-glucose	12±3**	9±1**
20 mM-glucose	100±23	100±16
20 mM-glucose + 500 nM-PMA	320±44**	93±7^{ns}
20 mM-glucose + 500 μM-CCh	266±32**	143±9*
20 mM-glucose + 5 mM-CCh	258±51**	139±10*

on insulin secretion were significantly decreased in PKC-depleted islets, as shown in Table 2. Thus, in control (4αPDD-treated) islets, 500 μM-CCh enhanced glucose (20 mM)-induced insulin secretion by 166±32% (mean±S.E.M., $n = 5$) of controls (20 mM-glucose alone), whereas in PKC-depleted (PMA-treated) islets CCh only increased secretion by 43±9% ($P < 0.02$ versus 4αPDD-treated). Note, however, that the decreased responses of PKC-depleted islets to CCh still represented a significant enhancement of insulin secretion ($P < 0.05$ versus paired 20 mM-glucose controls), whereas PMA pretreatment totally abolished any secretory response to a subsequent exposure to PMA (Table 2).

The decreased responsiveness of PKC-depleted islets to CCh did not appear to be due to impaired coupling of muscarinic receptors to phospholipase C, nor to direct inhibition of phospholipase C activity, since CCh stimulated production of [^{3}H]inositol-containing compounds to a similar extent in control islets and in islets in which PKC activity had been depleted. Compared with total radioactivity (c.p.m.) in [^{3}H]inositol-containing compounds eluted from the columns in the presence of 20 mM-glucose alone, [^{3}H]inositol-labelled 4αPDD-treated islets showed a 42±2% increase and PMA-treated islets a 53±4% increase in response to 500 μM-CCh (means±S.E.M., $n = 3$; $P > 0.05$). Thus, in the presence of 20 mM-glucose, CCh significantly stimulated the accumulation of IP_1 and IP_2 in both PMA-treated and 4αPDD-treated islets, as shown in Table 3. Despite this, full potentiation of glucose-induced secretion from PMA-treated islets was not achieved even in the presence of 5 mM-CCh, a supramaximal stimulatory concentration of this agonist (Table 2).

DISCUSSION

Pancreatic insulin secretion is enhanced *in vivo* by the release of ACh from parasympathetic fibres of the vagus nerve, which penetrate the islets and terminate close to the endocrine cells (see Miller, 1981, for review). ACh and its non-hydrolysable analogue, CCh, also potentiate glucose-stimulated insulin release *in vitro* (Malaisse *et al.*, 1967; Nenquin *et al.*, 1984; Hughes *et al.*, 1987). It is known that these cholinergic agonists exert their effects by binding to specific muscarinic M_2 receptors (Henquin & Nenquin 1988; Verspohl *et al.*, 1988) and that activation of these receptors, and potentiation of secretion, can be blocked by the muscarinic antagonist atropine (Malaisse *et al.*, 1967; Gagerman *et al.*, 1978; Mathias *et al.*, 1985). However, the sequence of events resulting from β-cell muscarinic-receptor occupation that culminates in enhanced insulin secretion is not well-defined.

Table 3. Inositol phosphate generation in normal and PKC-depleted islets

After labelling of islet phosphoinositides with [^{3}H]inositol for 2 h, CCh stimulated significant increases in the incorporation of ^{3}H into IP_1 and IP_2, in both 4αPDD- and PMA-treated islets: * indicates $P < 0.05$ versus appropriate 20 mM-glucose controls ($n = 3$ for all experiments). Basal unstimulated (2 mM-glucose) levels of IP_1 and IP_2 were higher in PMA-treated than 4αPDD-treated islets, perhaps reflecting a negative regulatory effect of PKC on phospholipase C in normal islets, which was relieved after PKC down-regulation. Exposure to PMA did not significantly affect ^{3}H uptake into islets: 3502±163 c.p.m./10 4αPDD-treated islets, 4733±906 c.p.m./10 PMA-treated islets ($P > 0.2$).

	Radiolabel incorporation (^{3}H c.p.m./250 islets)			
	Inositol	IP_1	IP_2	IP_3
4αPDD-treated				
2 mM-glucose	2044±163	674±46	207±18	29±2
20 mM-glucose	2355±213	853±89	187±24	30±2
20 mM-glucose + 500 μM-CCh	2549±27	1764±46*	519±13*	32±0.3
PMA-treated				
2 mM-glucose	1974±164	1220±4	275±11	30±2
20 mM-glucose	1917±62	1606±81	326±51	31±3
20 mM-glucose + 500 μM-CCh	2122±18*	3123±313*	651±176*	34±4

In common with other reports (Gagerman *et al.*, 1978; Mathias *et al.*, 1985; Garcia *et al.*, 1988), we found that insulin secretion was enhanced by a muscarinic agonist in a dose-dependent manner. However, whereas most other studies (Gagerman *et al.*, 1978; Nenquin *et al.*, 1984; Mathias *et al.*, 1985) reported effects of muscarinic agonists on secretion from freshly isolated islets, CCh did not consistently potentiate insulin release from rat islets under these conditions. Lack of response of freshly isolated islets to CCh may have been a result of β-cell muscarinic-receptor damage during collagenase isolation of islets, as suggested by Hughes *et al.* (1987). In contrast with the report by Meglasson *et al.* (1986), where ACh elevated only the first phase of 10 mM-glucose-induced insulin secretion, but in accordance with the studies by Nenquin *et al.* (1984) we found that CCh potentiated both phases of insulin release. Furthermore, the inability of CCh to increase insulin secretion significantly at sub-stimulatory glucose concentrations, observed both in static incubations and in perifusions, is in agreement with other reports that muscarinic agonists act as potentiators, rather than initiators, of insulin release (Nenquin *et al.*, 1984; Morgan *et al.*, 1985; Hughes *et al.*, 1987).

The major aim of the present study was to determine the involvement of PKC in the potentiation of insulin secretion by CCh. The stimulation of PKC translocation by CCh observed in the present experiments using normal rat islets is in accordance with recent reports of CCh-induced PKC translocation in insulin-secreting tumour cells (Yamatani *et al.*, 1988; Regazzi & Wollheim, 1988). The redistribution of PKC presumably reflects generation of membrane-associated DAG, and is consistent with the report that CCh generates DAG in islets by activation of phospholipase C, a response that is maximal by 1 min and maintained for at least 5 min (Peter-Riesch *et al.*, 1988). In the present study, both insulin release and PKC redistribution were stimulated by CCh at times when it would be expected to increase DAG production, suggesting that the translocation of PKC to membranes may be an early event in cholinergic enhancement of insulin release.

Prolonged exposure to tumour-promoting phorbol esters has been shown specifically to down-regulate total PKC activity in islets of Langerhans (Hii *et al.*, 1987; Metz, 1988) as well as other cell types (see Murray *et al.*, 1987), probably as a result of increased degradation of the enzyme without any change in its rate of synthesis (Young *et al.*, 1987). We have previously shown that incubation of islets with PMA for 5 min causes an increase in PKC activity associated with membrane fractions (Persaud *et al.*, 1989), and in this activated state the enzyme is susceptible to proteolytic cleavage by a Ca^{2+}-activated neutral protease (Nishizuka, 1984). In the present study, islets cultured overnight with 200 nM-PMA showed an approx. 90% loss of PKC activity associated with the cytosolic fraction. This is consistent with translocation of enzyme activity followed by progressive degradation of membrane-associated PKC until virtually all PKC that was originally present in the cytosol had been activated and proteolytically cleaved. Down-regulation of islet PKC resulted in a significantly decreased secretory response to CCh, even at concentrations as high as 5 mM. However, under these conditions of negligible PKC activity, with islets which were totally unresponsive to the secretagogue effects of PMA, CCh caused a lesser, but still significant, potentiation of glucose-stimulated insulin release. These results are in agreement with those of Malaisse & Sener (1985) in which H-7, an inhibitor of PKC activity, decreased, but did not abolish, the increment in insulin secretion in response to CCh at 8.3 mM-glucose.

Short-term exposure to PMA (5–15 min) has been reported to inhibit phospholipase-C-mediated hydrolysis of membrane phosphoinositides in many cell types, including insulin-secreting tumour cells (Biden *et al.*, 1988). This action is thought to be mediated by activation of PKC resulting in either a decrease in receptor number (Lynch *et al.*, 1985) or impaired G-protein–phospholipase-C coupling (Orellana *et al.*, 1987). However, such a negative-feedback effect of PKC activation cannot account for the decreased secretory response to CCh after treatment of islets with PMA for > 20 h in the present studies, because CCh was able to stimulate phospholipase-C activity in these islets, as assessed by increased generation of [^{3}H]inositol phosphates. In any case, PKC-mediated down-regulation of phospholipase-C activity as a result of PMA treatment would be expected to be a short-term effect, because PKC within islets is depleted in the continued presence of PMA. Thus any PKC-dependent inhibition of phosphoinositide hydrolysis should not be maintained.

The results of our experiments with PKC-depleted islets suggest that PKC is not the only mechanism through which CCh modulates glucose-stimulated insulin release, but that activation of this enzyme by CCh promotes a full potentiation of secretion. Several other observations suggest the involvement of a mechanism(s) other than PKC activation in the action of cholinergic agonists on β-cells. For example, in the present study, CCh did not initiate insulin secretion at 2 mM-glucose, despite its ability to induce redistribution of PKC activity at this sub-stimulatory glucose concentration, suggesting that activation and redistribution of PKC is insufficient to initiate insulin secretion. Similarly, in the absence of a stimulatory concentration of glucose, CCh stimulated mobilization of intracellular Ca^{2+} in rat islets, presumably as a result of inositol trisphosphate generation, but it did not stimulate insulin secretion under the same conditions (Morgan *et al.*, 1985).

In the mouse β-cell ACh augments insulin secretion only when the β-cell plasma membrane has been depolarized by glucose or other primary stimuli (Hermans *et al.*, 1987). It has therefore been proposed that ACh potentiates insulin release by increasing the β-cell membrane permeability to Na^+ ions, resulting in further membrane depolarization and enhanced influx of Ca^{2+} (Henquin *et al.*, 1988). However, further studies by Henquin and co-workers have shown that, at a supramaximal concentration of glucose (30 mM), ACh has only minor effects on Ca^{2+} influx and yet markedly augments the secretory response (Garcia *et al.*, 1988). Muscarinic agonists may therefore potentiate insulin secretion by at least two distinct mechanisms: an initiation by enhanced Ca^{2+} influx and a modulation of the magnitude of the response by activation of PKC.

Financial assistance from the British Diabetic Association (BDA) and the Nuffield Foundation is gratefully acknowledged. S. J. P. is a recipient of a Kings's College Junior Research Studentship. P. M. J. is an R. D. Lawrence Research Fellow of the BDA. D. S. is a Royal Society 1983 Research Fellow.

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Received 5 July 1989/21 August 1989; accepted 24 August 1989